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Journal of Chromatography B, 661 (1994) 346–350

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Sensitive high-performance liquid chromatographic assay with ultraviolet detection of methadone enantiomers in plasma

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First received 12 April 1994; revised manuscript received 26 July 1994

Abstract

Methadone is being prescribed increasingly as an analgesic in palliative medicine. *R*-Methadone has been shown to be responsible for most of the pharmacological activity of this drug. Despite this, in most countries it is administered as the racemate. Few assay methods for the enantiomers are available; even fewer can determine accurately the low concentrations of enantiomers required to undertake pharmacokinetic studies in patients taking the drug in analgesic doses. We present here an HPLC method used to determine concentrations of the specific enantiomers of methadone as low as 5.0 ng/ml with adequate precision and accuracy. The mean *R*/*S* ratio of the plasma concentrations was 0.80 ± 0.05 ($n = 3$ samples) in one patient taking 25–27.5 mg daily and 1.21 ± 0.12 ($n = 6$ samples) in another taking 10–20 mg daily. In the second patient, concentrations of the enantiomers ranged between 5.8 and 25.9 ng/ml. Tricyclic antidepressants did not interfere with the assay but dextropropoxyphene did. Its presence could be detected by dual wavelength monitoring.

1. Introduction

Methadone is used widely in programs for the treatment of drug addiction and increasingly to treat pain, particularly in the terminally ill. It is administered as the racemate but the *R*-enantiomer provides most of the pharmacological activity [1].

Nakamura et al. [2] reported a gas chromatographic–mass spectrometric method for the analysis of the enantiomers of methadone. This method required the use of stable isotopes [2]. Separation conditions for the enantiomers of methadone in pure solution have been reported for bonded α1-acid glycoprotein (AGP) [3] and β-cyclodextrin columns [4]. AGP columns have been utilised in an HPLC assay for the enantiomers of methadone in plasma [5] which was reported to have a limit of detection of 10 ng/ml with adequate precision at 100 ng/ml. It was demonstrated to be suitable for the analysis of samples from methadone maintenance subjects. Kristensen and Angelo [6] have also reported an enantioselective assay for the determination of methadone using gas chromatography. The

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0378-4347/94/$07.00
SSDI 0378-4347(94)00341-6

method had a limit of quantitation of approximately 25 ng/ml and involved extensive extraction and derivatisation. The chromatographic run time was 30 min.

We present here an HPLC method with adequate precision and accuracy at concentrations as low as 5 ng/ml and quantitative data in two patients taking low doses of the drug.

2. Experimental

2.1. Chemicals

HPLC-grade acetonitrile and methanol were obtained from Mallinckrodt (Paris, KY, USA) and triethylamine (Chromonorm for HPLC) from Rhone-Poulenc (Manchester, UK). Deionised water (18 MOhm) was obtained from a Milli-Q ultrapure water system (Millipore, Sydney, Australia) after pre-treatment using a Milli-RO reverse osmosis unit. Trimipramine maleate was obtained from Sigma (St. Louis, MO, USA). *R*-Methadone and *S*-methadone were obtained from Ultrafine Chemicals (Manchester, UK) by custom synthesis. *RS*-Methadone was a gift from Wellcome Australia (Sydney, Australia). Drug standards used to test for interferences were obtained from Sigma. All other chemicals were of analytical reagent grade.

2.2. HPLC

The HPLC system consisted of a Model 590 pump, Model 712 WISP autosampler, Model 484 ultraviolet detector (set at a wavelength of 210 nm), and a Model 820 Maxima Data reduction system (Millipore-Waters, Milford, MA, USA). The column was a Cyclobond Type I-Beta RSP (Astec, Whippany, NJ, USA). The mobile phase was prepared by mixing 200 ml of acetonitrile, 50 ml of methanol and 750 ml of 1% triethylamine which had been adjusted to pH 6.0 using orthophosphoric acid. The column was submerged in a refrigerated water bath to maintain a constant temperature of 18°C. The mobile phase flow-rate was 0.60 ml/min.

2.3. Procedure

Because the internal standard trimipramine maleate is adsorbed slowly to glass from dilute aqueous solution, it was prepared as a stock solution of 7 mg in 50 ml of methanol (0.1 mg/ml free base). Each day, 400 μl was diluted to 100 ml in deionised water.

To 15-ml culture tubes, 1 ml of sample, 0.5 ml of internal standard solution, 1 ml of 1 *M* sodium hydroxide and 10 ml of 2% butanol in heptane were added. The tubes were capped and rotated on a laboratory mixer for 20 min. After centrifugation at 500 *g* for 5 min, the organic layer was transferred to 15-ml tapered-base centrifuge tubes, each containing 200 μl of 0.005 *M* hydrochloric acid. After vortex-mixing (2 min) the samples were centrifuged again for 5 min at 500 *g*. The aqueous layer was transferred to respective autosampler vials from which 100-μl injections were made. A standard curve was included in each batch of samples. The line of best fit for the standards was determined using linear regression incorporating a weighting factor of 1/concentration squared. Concentrations of unknowns were then determined by inverse prediction from the line of best fit.

2.4. Linearity

RS-Methadone was added to human serum that was devoid of interfering drugs to prepare standards containing 2.3, 9.1, 45, 136 and 272 ng/ml of each of the enantiomers. These standards were included in each batch of samples and the standard curve was plotted as the peak-height ratio of the respective enantiomer to the internal standard. To assess linearity, the line of best fit was determined by least squares regression.

2.5. Precision

To determine precision, a further three samples were prepared using serum from a second subject to which was added 4.5, 27.2 and 227 ng/ml of each of the enantiomers. Each was assayed in quadruplicate on four separate days.

Analysis of variance was used to determine the within-day, between-day and total coefficients of variation [7].

2.6. Accuracy

Accuracy of the assay was assessed by expressing the mean assayed concentration obtained for the precision samples as a percent of the weighed-in concentration.

2.7. Recovery

Each time the precision samples were assayed, duplicate 100-μl injections of 193 ng/ml pure standard in 0.005 *M* hydrochloric acid were also chromatographed (prepared from the stock solution used to prepare the precision samples). Recovery was determined from the ratio of the peak height for each enantiomer expressed as a percent of the expected peak height. This was determined from the mean height of the pure standard injections.

The expected peak height for the internal standard was calculated from the mean height of duplicate 100-μl injections of the internal standard working solution. Recovery of the internal standard was then expressed as the percent of the actual peak height relative to that expected.

2.8. Assessment of interferences

The assay was assessed for chromatographic interference from the tricyclic antidepressants amitriptyline, imipramine, nortriptyline and desipramine. Weighed-in concentrations of 500 ng/ml of each of the tricyclic antidepressants were added to blank serum and the sample assayed. Dextropropoxyphene is known to interfere in some assays for *RS*-methadone and was tested for chromatographic interference by injection of pure standard solution.

2.9. Clinical studies

Several serum samples were obtained from each of two males receiving oral methadone for pain relief from severe burns. The first patient was 31 years old and the second 59.

3. Results and discussion

Fig. 1 shows chromatograms of an extract from a blank serum sample without the internal

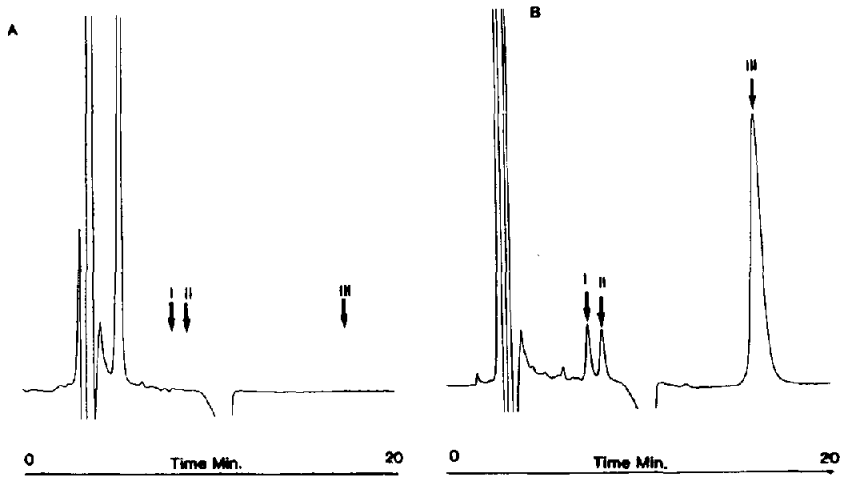

Fig. 1. Chromatograms of (A) extract of blank plasma without addition of internal standard, and (B) extract of blank plasma after addition of 50 ng/ml of *RS*-methadone. Peaks: I = *R*-methadone, II = *S*-methadone, and III = internal standard. Chromatograms were obtained at 210 nm and 0.02 AUFS.

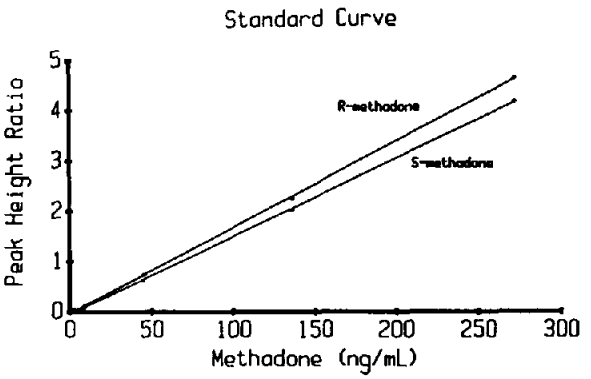

Fig. 2. Standard curve of *R*-methadone (upper line) and *S*-methadone (lower line). The regression equation for *R*-methadone was $y = 0.017x - 0.0209$ and for *S*-methadone, $y = 0.0154x - 0.0382$. Correlation coefficients for both enantiomers were 0.999.

Table 2
Recovery data

Weighed-in concentration (ng/ml)	Enantiomer	Recovery (mean ± S.D.) (%)
2.4	*R*	79.8 ± 9.6
	S	84.2 ± 9.4
48.3	*R*	83.2 ± 7.5
	S	88.2 ± 8.0
290	*R*	78.4 ± 3.6
	S	84.3 ± 4.0

Recoveries (%) are expressed as the actual assayed peak height relative to the expected height from direct injections of pure standards ($n = 6$). Recovery for the internal standard at concentrations used in the assay was 74.7% with a standard deviation of 2.5%.

standard, and the same sample extracted using internal standard after adding 25 ng/ml of *RS*-methadone. Fig. 2 shows the linear standard curves for *R*-methadone and *S*-methadone.

Precision and accuracy data are summarised in Table 1. Based on the data obtained for the weighed-in concentration of 4.5 ng/ml, the lower limit of quantitation for each enantiomer was 5.0 ng/ml. This was based on a total coefficient of variation (C.V.) less than 15% and an absolute accuracy less than 0.5 ng/ml (i.e. a relative accuracy less than 10%). The limit of detection determined as three times the baseline noise was 1.0 ng/ml. Recovery data, presented in Table 2, indicate adequate recovery given that these data incorporate transfer losses during extraction.

Chromatograms of the extracts of weighed-in tricyclic antidepressant samples showed no interference. On the other hand, the peak for dextropropoxyphene was superimposed on the *R*-methadone peak. Moreover, the drug was extracted from serum using the procedure described here. Therefore, co-administration of dextropropoxyphene should be avoided when using this method. To detect inadvertent co-administration, we assessed the utility of dual wavelength monitoring at 210 and 230 nm. The

Table 1
Precision and relative accuracy data

Weighed-in Concentration (ng/ml)	Enantiomer	Mean assayed concentration (ng/ml)	Precision (%)			Accuracy (%)
			Between-run	Within-run	Total	
4.5	*R*	4.00	8.8	8.7	12.4	88
	S	4.02	13.0	7.6	15.0	88
27.2	*R*	25.6	3.1	5.4	6.2	94
	S	25.3	2.2	6.1	6.4	93
227	*R*	250	3.2	2.8	4.2	110
	S	252	2.3	2.9	3.7	111

Precision is expressed as the coefficient of variation (%) and accuracy as the assayed concentration, relative to the weighed-in concentration (%) ($n = 16$ in all cases).

Table 3
Summary of assay results for morning-through samples from patients treated with analgesic doses of *RS*-methadone

Day	*R*-methadone (ng/ml)	*S*-methadone (ng/ml)	*R*/*S* ratio	Dose (mg)	
				AM	PM
Patient 1					
1	–	–	–	10	15
2	19.2	15.3	0.80	10	17.5
5	17.8	20.9	0.85	10	17.5
6	16.3	21.7	0.75	–	–
Patient 2					
1	–	–	–	5	5
2	6.0	5.8	1.03	5	5
3	8.8	6.6	1.33	10	10
4	14.2	13.1	1.08	10	10
5	19.4	15.9	1.22	10	10
6	21.5	16.5	1.30	10	10
7	25.9	20.3	1.28	–	–

Patient 1 was a 31 year old male and patient 2 was a 59 year old male. Day 2 is the day the first sample was obtained, not the second day of methadone treatment.

ratio of peak heights at 210 to 230 nm was 10.4 for dextropropoxyphene and 3.6 for *R*-methadone. Dual wavelength monitoring at 210 and 230 nm would be useful to detect inadvertent co-administration of dextropropoxyphene.

Table 3 summarises the patient data obtained using this method. They demonstrate that sensitive methods are required if pharmacokinetic studies of the enantiomers are to be undertaken in patients receiving *RS*-methadone for analgesia. The enantiomeric ratios are consistent with those of other studies [5,6,8].

To date, several hundred injections have been made using the Cyclobond column and no loss of resolution is yet evident. Our experience with bonded α1-acid glycoprotein columns has indicated that their life-time is substantially shorter than this. Thus, the improved column life obtained with this present method is advantageous, as is the sample extraction procedure and shorter run time.

Acknowledgements

We are grateful for funding by the Princess Alexandra Hospital Research and Development Foundation to purchase *R*-methadone and *S*-methadone.

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